JOURNAL OF CLINICAL MICROBIOLOGY, May 2011, p. 1750–1757 Vol. 49, No. 5
0095-1137/11/$12.00 doi:10.1128/JCM.02348-10

Discrimination of Major Capsular Types of *Campylobacter jejuni* by Multiplex PCR▽†

Frédéric Poly,[1] Oralak Serichatalergs,[2] Marc Schulman,[1] Jennifer Ju,[1] Cory N. Cates,[3] Margaret Kanipes,[3] Carl Mason,[2] and Patricia Guerry[1]*

Naval Medical Research Center, Silver Spring, Maryland[1]; Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand[2]; and Department of Chemistry, North Carolina Agricultural and Technical University, Greensboro, North Carolina[3]

Received 22 November 2010/Returned for modification 3 January 2011/Accepted 9 March 2011

The polysaccharide capsule (CPS) of *Campylobacter jejuni* is the major serodeterminant of the Penner serotyping scheme. There are 47 Penner serotypes of *C. jejuni*, 22 of which fall into complexes of related serotypes. A multiplex PCR method for determination of capsule types of *Campylobacter jejuni* which is simpler and more affordable than classical Penner typing was developed. Primers specific for each capsule type were designed on the basis of a database of gene sequences from the variable capsule loci of 8 strains of major serotypes sequenced in this study and 10 published sequences of other serotypes. DNA sequence analysis revealed a mosaic nature of the capsule loci, suggesting reassortment of genes by horizontal transfer, and demonstrated a high degree of conservation of genes within Penner complexes. The multiplex PCR can distinguish 17 individual serotypes in two PCRs with sensitivities and specificities ranging from 90 to 100% using 244 strains of known Penner type.

Campylobacter jejuni is one of the major causes of human bacterial diarrheal disease (29), with an estimated annual incidence of 400 million worldwide. The disease is zoonotic, with wild and domesticated poultry representing major reservoirs (13, 47). The symptoms of campylobacteriosis can range from a mild, watery diarrhea to dysentery, often with abdominal pain and fever (1). Moreover, there are several serious postinfectious complications associated with *C. jejuni*, including reactive arthritis (38), Guillain-Barré syndrome (2), and irritable bowel syndrome (35, 44).

The polysaccharide capsule (CPS) represents an interface between the bacterium and the environment and contributes to virulence. Noncapsulated mutants of *C. jejuni* were demonstrated to have reduced abilities to invade INT407 cells *in vitro* (6), to colonize chickens (5, 12), and to cause diarrheal disease in ferrets (6). CPS also contributes to resistance to normal human serum (6). The CPS is phase variable in expression, presumably due to slip-strand mismatch repair in genes essential for CPS synthesis, a mechanism of variation that is common in *C. jejuni* (14, 31, 45). The ability to turn CPS expression on and off suggests that there may be advantages to the pathogen to express this polysaccharide coat only at specific times during its life cycle. Importantly, a prototype CPS conjugate vaccine against *C. jejuni* 81-176 provided 100% efficacy against diarrheal disease in a nonhuman primate model, further implicating this structure as both a virulence determinant and a protective antigen (27).

The genes for CPS biosynthesis are located in one of the more hypervariable regions of the *C. jejuni* genome. The high degree of variability of CPS genes is consistent with CPS being the major serodeterminant of the Penner or heat-stable (HS) serotyping scheme (19). However, in some serotypes lipooligosaccharide (LOS) has been shown to play a role in Penner serospecificity (39). The Penner scheme is a passive slide hemagglutination assay that includes 47 *C. jejuni* serotypes (33, 34). Twenty-two of the 47 serotypes fall into complexes of what appear to be structurally related CPS types (39). Currently, the DNA sequences of 10 *C. jejuni* hypervariable CPS loci (HS1, HS19, HS23, HS36, HS23/36, HS41, HS2, HS6, HS4/13/64, HS53) have been reported (9, 10, 18, 31, 32), and these loci range in size from 15 to 34 kb. The genes in the variable CPS loci include genes encoding sugar biosynthetic enzymes, putative glycosyl transferases, and genes of unknown function. Genes involved in heptose biosynthesis are conserved in the CPS loci of many strains. These genes include *hddC*, a putative heptose transferase; *gmhA2*, a phosphoheptose isomerase; *hddA*, a putative D-*glycero*-D-manno-heptose 7-phosphate kinase; and *dmhA*, a GDP-mannose 4,6-dehydratase responsible for the conversion of heptose to deoxyheptose. The genes for biosynthesis of *O*-methyl phosphoramidate (MeOPN), an unusual modification on some *C. jejuni* capsules, were initially identified and characterized in *C. jejuni* NCTC 11168 (23). These highly conserved MeOPN biosynthesis genes have been found in about 70% of the strains sequenced to date. Similarly, McNally et al. identified two distinct MeOPN transferases in NCTC 11168 responsible for addition of MeOPN to distinct sugar moieties in the NCTC 11168 CPS (23). There is variability in the predicted protein sequences of MeOPN transferases among sequenced strains, consistent with attachment of MeOPN to different sugars in different CPS structures. The MeOPN transferases also appear to undergo phase variation in expression, which contributes to the observed nonstoichiometric levels of MeOPN on CPS. Phase variations affecting capsule structure appear to modulate the Penner serotype within the HS23/36 complex. HS23 and HS36 strains and strains that

* Corresponding author. Mailing address: Enteric Diseases Department, Naval Medical Research Center, 503 Robert Grant Ave., Silver Spring, MD 20910. Phone: (301) 319-7662. Fax: (301) 319-7679. E-mail: patricia.guerry@med.navy.mil.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 16 March 2011.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MAR 2011		**00-00-2011 to 00-00-2011**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Discrimination of Major Capsular Types of Campylobacter jejuni by Multiplex PCR	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Naval Medical Research Center,503 Robert Grant Avenue,Silver Spring,MD,20910**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**8**	

TABLE 1. Strains used in this study

Strain	Penner type	Reference or source
ATCC 43429	HS1 (type)	34
ATCC 43430	HS2 (type)	34
NCTC 11168	HS2	31
ATCC 43431	HS3 (type)	34
ATCC 43432	HS4 (type)	34
BH-01-0142	HS3/13/50	37
GC8486	HS4/13/64	36
ATCC 43433	HS5 (type)	34
81116	HS6	30
ATCC 43436	HS8 (type)	34
ATCC 43438	HS10 (type)	34
ATCC 43441	HS13 (type)	34
ATCC 43442	HS15 (type)	34
ATCC 43444	HS17 (type)	34
ATCC 43446	HS19 (type)	34
ATCC 43448	HS22 (type)	34
81-176	HS23/36	20
ATCC 43478	HS28 (type)	34
ATCC 43460	HS41 (type)	34
ATCC 43461	HS42 (type)	34
ATCC 43463	HS44 (type)	34
ATCC 43465	HS50 (type)	34
RM1221	HS53	25
ATCC 49302	HS64 (type)	34

serotype as both HS23 and HS36 (e.g., *C. jejuni* strain 81-176) all express a capsule composed of repeating units of α-D-galactose, β-D-GlcNAc, and D-glycero-D-altro-heptose or deoxyheptose variants with and without methyl groups (4). The difference in serotype (HS23, HS36, or HS23/36) is due to phase variations in the genes encoding heptose synthesis and the MeOPN transferase (4, 18).

The Penner serotyping scheme was used extensively for epidemiological studies for many years, but the complexities and expense of producing the antisera specific to 47 *C. jejuni* serotypes have limited its usefulness. Other methods have largely replaced Penner typing for general epidemiological studies, such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and ribotyping. Given the recent data demonstrating that a CPS conjugate vaccine can protect against *C. jejuni* diarrhea in a primate model (27), we are interested in evaluating the feasibility of a multivalent CPS conjugate approach to prevent *C. jejuni* infections. Critical to this end is the determination of the valency that would be required for an effective vaccine in areas of the world where *C. jejuni* is endemic or hyperendemic. To address this, we have developed a multiplex PCR method that can rapidly determine CPS types. The primers were designed on the basis of published CPS loci from 10 serotypes and another 8 serotypes that were sequenced as part of this work. Here, we report the sequences of the CPS loci of these 8 serotypes and describe a multiplex PCR system that can accurately predict 17 major CPS types of *C. jejuni*. We also provide new information on the relationship of serotypes within some additional Penner complexes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used for sequence analysis and for development of the multiplex PCR are shown in Table 1. Collections of clinical isolates from Thailand and Egypt were used to validate the multiplex PCR. The Thai isolates were obtained from cases and asymptomatic controls in an acute diarrhea study among foreign travelers at Bumrungrad Hospital, Bangkok, Thailand, during 2001 and 2002, as well as *C. jejuni* isolates collected from U.S. soldiers with acute diarrhea and from asymptomatic controls during Operation Cobra Gold exercises at different sites in Thailand from 1998 to 2003. The Thai isolates were serotyped by Helen Tabor at the Canadian Reference Lab in Winnipeg, Manitoba, Canada. The Egyptian isolates were from a longitudinal village-based study of diarrhea in rural Egyptian children (41) and were serotyped by Eva Nielsen at the Danish Veterinary Laboratory, Copenhagen, Denmark. *C. jejuni* strains were routinely cultured at 37°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) on Mueller-Hinton (MH) agar plates.

DNA purification. *C. jejuni* genomic DNA was extracted from 16-h cultures following the method described by Sambrook et al. (43).

CPS sequencing strategies. Due to the large size of the CPS regions (15 to 35 kb), primers were designed in the highly conserved heptose genes *hddA* (hddA-L, 5′-GAAAGAGAAGATTTAGGCATAGTAGG; hddA-R, 5′-TCCATGATTTAACCCCCTCTT) and *dmhA* (dmhA-L, 5′-GGATTTACAGGGCAAGTTGG; dmhA-R, 5′-TTCTTGTAACAAAAGTGCGAATG). If the strains to be sequenced produced a positive amplification with primers hddA-L/R and dmhA-L/R, these genes were used as anchors for long-range PCR. In this case, primer kpsC-R (5′-GGATTTTCTTTTATGGCATCTTTT) was used with primer hddA-L and primer dmhA-R was used with primer kpsF-F (5′-AGTCGATGCTGATGCTATGG). This two-step PCR increases the probability of amplification by lowering the size of the PCR product. PCRs were performed using a MasterAmp Extra-Long PCR kit from Epicentre or LongAmp *Taq* DNA polymerase from New England BioLabs, following the manufacturers' protocols. PCRs with primers kpsC-R/hddA-L were performed at an annealing temperature of 55°C for 40 s and dmhA-R/kpsF-F at an annealing temperature of 56°C for 40 s. Both reactions were performed with a denaturing step at 94°C for 40 s and amplification of 10 min at 72°C for 25 cycles. PCR products were used to construct a genomic library using the TOPO cloning system (Invitrogen), following the manufacturer's protocol. Sequencing reactions were performed using an ABI Prism BigDye Terminator cycle sequencing kit and were purified and sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled using Sequencher (version 4.8) software (Gene Codes). Additional primers were designed to fill gaps or to correct potential errors or low-quality sequences, as needed. Artemis software (42) was used to annotate the CPS locus.

PCR primer design. A database of 18 CPS loci was created to identify unique regions of each serotype, and PCR primers were designed using the online software MuPlex MultiObjective multiplex PCR (40). Primers were designed with the following parameters: length between 18 and 30 residues, 20 to 50% GC content, and melting temperature ranging from 57 to 63°C. Primer sequences were further verified for absence of dimerization or hairpin formation using the web-enabled AutoDimer interface (46). The primer sets were designed in two mixes, alpha and beta, to produce amplicons that differ by at least 20 bp from the other amplicons in the same mix.

Multiplex PCR. PCRs were performed using AmpliTaq DNA polymerase FS (Perkin-Elmer–Applied Biosystems) and a 2720 thermal cycler. Alpha and beta mixes are used to obtain a final concentration of 3.25 ng/μl of each primer in the PCR. All PCRs were performed using the following parameters: 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s for a total of 29 cycles. The PCR products were analyzed by gel electrophoresis on 15-cm-long 2% agarose gels in 0.5× TBE (Tris-borate-EDTA) buffer at 175 V for 75 min. The sizes of the PCR products and corresponding serotype were determined by comparison with a 100-bp molecular size standard (New England BioLabs).

Nucleotide sequence accession numbers. The accession numbers for the CPS sequences described in this paper are shown in Table 2.

RESULTS AND DISCUSSION

Selection of strains for additional CPS locus sequencing. Strains were selected for CPS sequencing on the basis of limited information on serotypes common in the Armed Forces Research Institute of Medical Sciences (AFRIMS) collection. This included 5 serotypes for which no CPS sequence information existed: HS10, HS15, HS42, and the complex composed of HS8 and HS17. We compared these sequences to those of other CPS loci for the presence of conserved genes (e.g., heptose, fucose, and MeOPN biosynthesis genes). We

TABLE 2. Summary of sequenced *C. jejuni* CPS loci

Penner type	Size (bp)	GenBank accession no.	GC (%)	No. of genes	No. of MeOPN transferases	Heptose	Deoxyheptose	No. of sugar transferases	CPS structure available
HS1	15,180	BX545859[a]	26.8	11	1	No	No	3	22
HS2	34,180	AL111168.1[b]	26.5	28	2	Yes	No	8	18
HS3	26,371	HQ343268[f]	27.3	23	1	Yes	Yes	10	3
HS3/13/50	26,371	HQ343267[f]	27.3	23	1	Yes	Yes	10	Monteiro et al. (unpublished)
HS4	22,836	HQ343269[f]	28.0	18	2	Yes	Yes	4	Monteiro et al. (unpublished)
HS4/13/64	23,423	AASY01000000[c]	28.0	18	2	Yes	Yes	4	9
HS6	26,729	NC_009839[d]	27.6	23	0	No	No	8	28
HS8	22,063	HQ343270[f]	27.1	18	0	Yes	Yes	5	Monteiro et al. (unpublished)
HS10	27,307	HQ343271[f]	27.1	25	1	Yes	Yes	4	Monteiro et al. (unpublished)
HS15	23,868	HQ343272[f]	28.3	22	1	Yes	Yes	5	Monteiro et al. (unpublished)
HS17	22,064	HQ343273[f]	27.1	18	0	Yes	Yes	5	Monteiro et al. (unpublished)
HS19	16,727	BX545860[a]	26.1	13	1	No	No	4	21
HS23	24,627	AY332625[a]	27.0	21	1	Yes	Yes	7	4, 18
HS36	24,625	AY332624[a]	26.9	21	1	Yes	Yes	7	4, 18
HS23/36	24,625	BX545858[a]	27.1	21	1	Yes	Yes	7	4
HS41	34,118	BX545857[a]	27.2	30	0	Yes	Yes	2	16
HS42	23,268	HQ343274[f]	26.9	21	0	Yes	Yes	7	Monteiro et al. (unpublished)
HS53	18,272	CP000025.1[e]	27.0	15	0	Yes	Yes	7	11

[a] Karlyshev et al., 2005 (18).
[b] Parkhill et al., 2000 (31).
[c] Poly et al., 2007 (36).
[d] Pearson et al., 2007 (32).
[e] Fouts et al., 2005 (10).
[f] This study.

also examined the sequence of the CPS loci of strains from two major Penner complexes, HS3 and HS4. In this case we compared the sequences of the type strains of HS3 and HS4 to other published sequences from strains with mixed serotypes within each complex in an attempt to clarify the relationship among strains within these complexes. The overall data are summarized in Fig. 1 and Table 2. Actual open reading frames (ORFs) for each stain sequenced are shown in Table S1 in the supplemental material.

CPS loci of HS3 type strain and an HS3/13/50 strain. The CPS loci of the HS3 type strain and an HS3/13/50 strain (BH-01-142) are very similar. Most of the predicted proteins encoded by the genes in the CPS loci of HS3 and HS3/13/50 show 100% identity; only minimal amino acid variation (≥99.3% identity) was seen in the predicted products of two genes (see Table S1 in the supplemental material). In all previously sequenced CPS loci, all of the genes were found on the same strand (the negative strand, based on the genome sequence of NCTC 11168). However, three genes in HS3 and HS3/13/50, HS3.18, HS3.19, and HS3.20, are encoded on the other strand. Both strains contain genes for MeOPN synthesis and a single predicted MeOPN transferase gene that contains a homopolymeric tract capable of phase variation (Fig. 1; see Table S1 in the supplemental material). Both strains contain genes for heptose and deoxyheptose synthesis.

The structure of the CPS of the HS3 type strain is a repeating disaccharide of Gal and LD-*ido*-Hep [LD-*ido*-Hep-(1 → 4)-Gal-(1 → 3)]$_n$ (3). The structure of the CPS of the HS3/13/50 strain is being determined (M. A. Monteiro, personal communication), but composition analyses have indicated that this CPS is composed of Gal, 6-deoxy-*ido*-Hep (6d-*ido*-Hep), and small amounts of LD-*ido*-Hep (37). Moreover, ^{31}P nuclear magnetic resonance detected the presence of MeOPN on the CPS of HS3/13/50 (37); MeOPN was not reported on the CPS of the HS3 type strain, likely due to differences in techniques used in this earlier study. Since the LOS cores of these two strains are identical (3, 37), it is likely that the presence of 6d-*ido*-Hep in HS3/13/50 CPS is responsible for the mixed serotype.

Comparison of CPS loci of HS4 type strain and an HS4/13/64 strain. The CPS locus of the HS4 type strain was very similar to that of a strain that serotyped as HS4/13/64 (CG8486) (9) (Fig. 2A). In both strains, all genes except one, HS4.16, were encoded on the negative strand. The polysaccharide structure of the CPS of the type strain of HS4 has not been determined, but that of HS4/13/64 has been shown to be a repeating disaccharide of 4-substituted *N*-acetyl-β-D-glucopyranosamine and 3-substituted 6-deoxy-β-D-*ido*-heptopyranose, with MeOPN attached to both the O-2 and O-7 positions of the heptopyranose residue (9). At the DNA level, both the HS4 type strain and HS4/13/64 have genes for heptose, deoxyheptose, and MeOPN synthesis, as well as two putative MeOPN transferase genes (Fig. 1; see Table S1 in the supplemental material). The predicted proteins encoded by the CPS genes of HS4/13/64 and the HS4 type strain show >92% identity, with the exception of the two putative MeOPN transferases, HS4.07

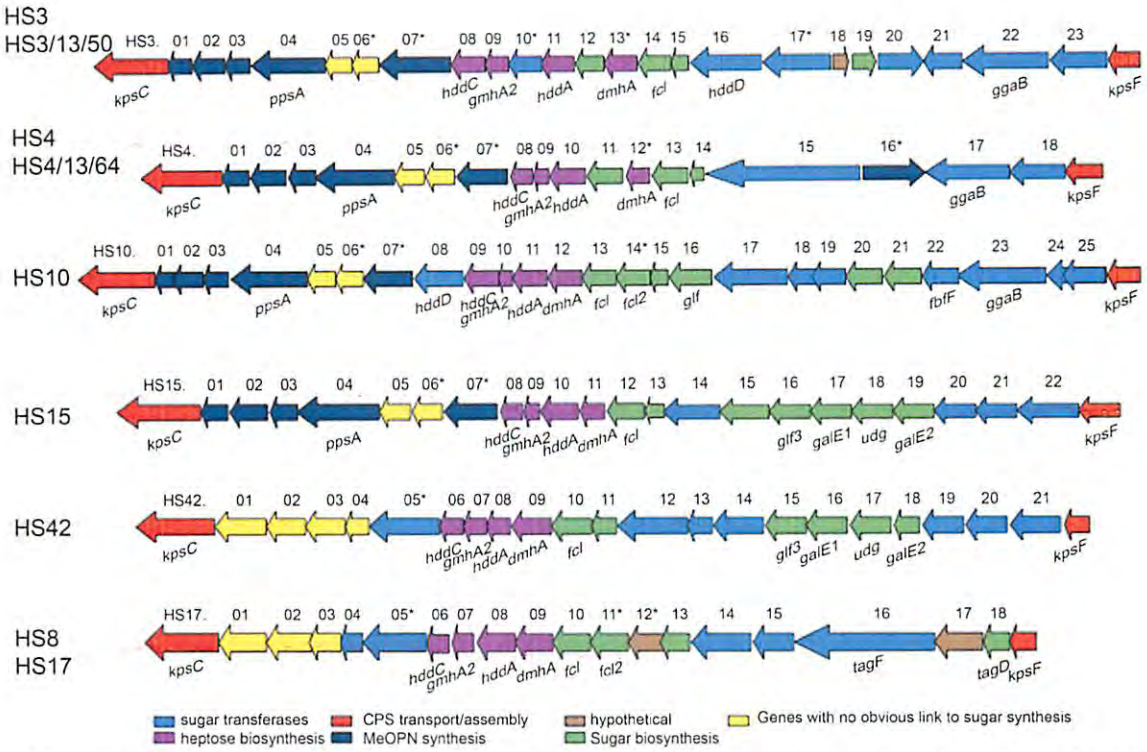

FIG. 1. Schematic of variable CPS loci from representative Penner serotypes. CPS loci were sequenced from the strains listed in Table 1. Gene names were attributed if the predicted protein showed >80% sequence identity with other known *C. jejuni* proteins. Genes are color coded as shown in the key to the figure on the basis of best homology to any predicted protein in databases by BLAST analyses. Genes containing homopolymeric tracts of >8 G or C residues are marked with an asterisk.

and HS4.16 (Fig. 2A). HS4/13/64 contains two MeOPN transferases (Cj8486_1464c and Cj8486_1475c), consistent with the presence of MeOPN on either the O-2 or O-7 position of the heptose (9). In contrast, in the type strain of HS4, one of these MeOPN transferases (HS4.07) is truncated, suggesting that the HS4 CPS contains MeOPN in a single position. Structural studies to examine this are under way (M. A. Monteiro, personal communication).

CPS sequencing of HS10. The HS10 locus appeared to be a mosaic of HS41 (18) and the HS4 complex (9) (Fig. 2B). The sequence revealed the presence of MeOPN, heptose, deoxyheptose, and fucose biosynthesis genes. HS10 contains 8 unique genes, including a single MeOPN transferase gene.

CPS sequence of HS15. The HS15 CPS locus also appeared, like that of HS10, to be a mosaic between HS41 and the HS4 complex, as shown in Fig. 2C. The genes from *kpsC* to *hddA* show a high degree of similarity with those in the HS4/13/64 CPS locus, including one of the two MeOPN transferases (see Table S1 in the supplemental material). The last seven genes at the right end of the HS15 CPS locus are conserved with the corresponding region of HS41. The CPS of HS41 contains arabinose (16), and the similarity of HS41 to HS15 in this region might suggest that the CPS of HS15 also contains arabinose. The CPS structure of HS15 is being determined (M. A. Monteiro, personal communication).

HS42 CPS sequencing. The HS42 CPS locus is related to the HS41 locus (Fig. 1 and Fig. 2D; see Table S1 in the supplemental material), including the putative genes for arabinose incorporation into CPS. The major differences are that 4 unique putative glycosyl transferases that are not found in HS41 are present in HS42 and that HS42 lacks 12 genes found in HS41. The capsule locus of HS42, like that of HS41, lacks genes for MeOPN synthesis but contains conserved genes involved in heptose and deoxyheptose biosynthesis.

HS17 and HS8 CPS loci. HS8 and HS17 form another Penner complex, and DNA sequence analysis of their CPS loci indicates that they are virtually identical to each other and are a mosaic of other CPS types. Both strains contain genes involved in heptose and deoxyheptose synthesis but lack genes for MeOPN. The only major difference between the CPS loci of HS8 and HS17 is in *orf16*, which encodes a putative CDP glycerol-phosphotransferase. In HS8, this ORF encodes a predicted protein of 1,619 amino acid residues, and in HS17 the protein is 1,578 amino acids. The truncation occurs at a homopolymeric tract of 6 T residues in HS8, whereas the homopolymeric tract is 7 T residues in HS17, which results in a premature truncation in HS17. The significance of this difference is not known, but it may reflect slip-strand mismatch repair (17).

There is considerable sequence identity of genes located between *kpsC* and *gmhA2* in HS41 and those in HS8 and HS17 (Fig. 2D), although the HS41.03 and HS41.04 ORFs are fused together in HS8 and HS17 (HS8.02). This difference is not due to a homopolymeric tract. HS8.07 to HS8.13 includes heptose and fucose synthesis genes. HS8.16, HS8.17, and HS8.18 ap-

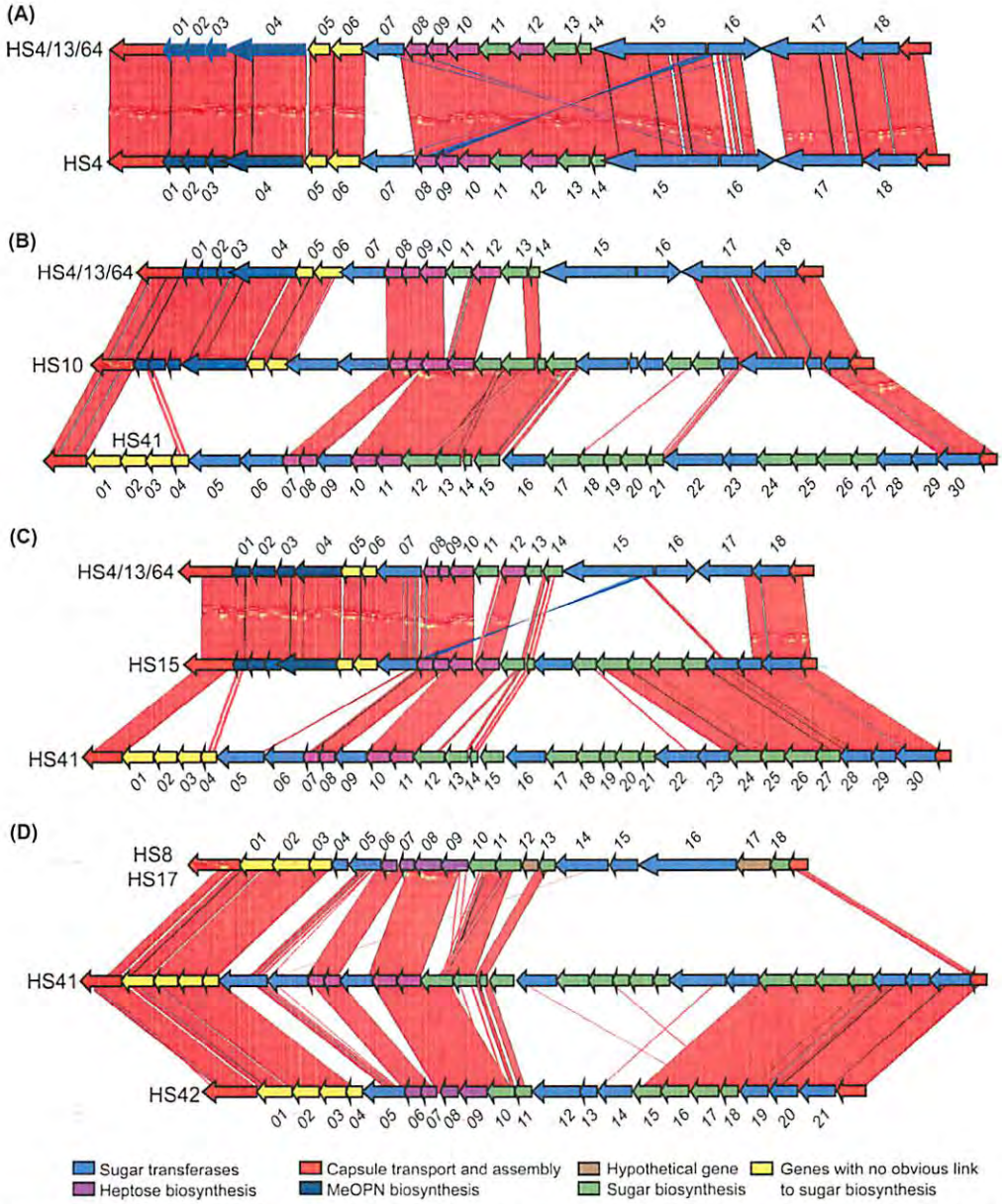

FIG. 2. Comparison of sequences of CPS loci of *C. jejuni* strains. (A) Comparison of the sequences of the CPS loci of *C. jejuni* HS4/13/64 (strain GC8486) and the HS4 type strain; (B) comparison of the CPS loci of *C. jejuni* HS4/13/64 (strain GC8486), the HS10 type strain, and a strain of HS41 (strain 176.83) (17); (C) comparison of the CPS loci of *C. jejuni* HS4/13/64 (strain GC8486), the HS15 type strain, and an HS41 strain (strain 176.83); (D) comparison of the CPS loci of strains *C. jejuni* HS41 (strain 176.83) and the type strains of HS8, HS17, and HS42. Vertical bars represent identical regions between loci. The red bars correspond to regions that are oriented similarly, and blue bars indicate regions oriented in opposite directions. The outermost genes in red represent *kpsC* (left) and *kpsF* (right), two conserved genes in CPS synthesis that bracket the hypervariable CPS loci. The comparison was made using the Artemis comparison tool (ACT).

pear to be related to the HS1 locus (see Table S1 in the supplemental material).

Design of multiplex PCR. Table 2 summarizes data for all sequenced CPS loci, including the 8 sequenced as part of this study. A database containing sequences of all available CPS loci was created to identify unique regions of each serotype. This data set included a partial sequence of the type strain of HS44, which forms a complex with HS1. The full sequence of HS44 will be presented with the HS44 CPS structure (M. A. Monteiro et al., unpublished data). Two primer sets were designed for the HS4 complex, and these are based on differences in MeOPN transferases among the sequenced strains in this complex (see above). This decision was based on an assumption that the differences among the members of the HS4 complex are due to differences in the position of MeOPN on the polysaccharide, a hypothesis that is being investigated (Monteiro et al., unpublished). These primer sets, named Mu_HS4 and Mu_8486, respectively, were designed in HS4.07 and Cj8486_1475, both putative MeOPN transferases. Since some HS4 strains contain two MeOPN transferases, a strain can

TABLE 3. Multiplex PCR primers for determination of CPS type

Mix	Product size (bp)	Penner type recognized	Gene in which primer was designed	Forward sequence	Reverse sequence
Alpha					
Mu_HS2	62	HS2	Cj1437c	CAGCATTGGAGGATTTACAATATAT	CATCCTAGCACAACTCACTTCA
Mu_HS3	149	HS3	HS3.17	GGTAAGGTTGATTCTGGGTTTAAT	AGATTAGGCCAAGCAATGATAA
Mu_HS4	370	HS4 A	HS4.07	TATATTTGGTTAGGGATCCA	CCTAACATATCATACACTACGGT
Mu_HS6	185	HS6	C8J_1331	CATACATTTGCTTTCAGATTCTTTAC	ACACGCCTATTGTTGTTGTTC
Mu_HS10	229	HS10	HS10.08	TCTTATGCAGCACGCTGAT	CAAATTCAATCGACTAGCCACT
Mu_HS15C	325	HS15 and HS31	HS15.12	ACAGGTAATAAAATGTGCGAGTTT	ATGCATCTGCAACATCATCC
Mu_HS41	279	HS41	HS41.22c	CTTACATATGCTGGTAGAGATGATATG	TGCAATCTCTAAAGCCCAAG
Mu_HS53	251	HS53	CJE1602	AGGCAAGCAGGAATTGTTT	TTAATTGCTCTTTGGCAATCTT
Beta					
Mu_HS1D	607	HS1 complex	HS1.08	TTGGCGGTAAGTTTTTGAAGA	GCAAGAGAAACATCTCGCCTA
Mu_HS17	342	HS8 and HS17	HS17.16	TTCACGTGGAGGATTATTGG	TTGAACATTTCATGTGTATTCCCTA
Mu_8486	652	HS4 B	Cj8486_1475	GTGGACATGGAACTGGGACT	AAAACGTTTAAAGTCAGTGGAAA
Mu_HS23	161	HS23/36	CJJ81176_1435	GCTTGGGAGATGAATTTACCTTTA	GCTTTATATCTATCCAGTCCATTATCA
Mu_HS42E	441	HS42	HS42.14	ATGGTAAAACCGGCATTTCA	ATGCTTCAGTTCCACCCAAA
Mu_HS44	148	HS44	Not annotated	AGAAGATGCACTAGGCTCTAG	GCTATCTAATTCCATCCCTG

theoretically be positive with both Mu_8486 and Mu_HS4, and two such clinical isolates were identified (see below). Since the CPS loci of HS8 and HS17 are so similar (see above), a single primer set was designed for this complex. Although CPS has been shown not to be the serodeterminant of the HS6 serotype (18), the CPS gene sequences in this strain do not match any of the other published sequences, and a primer set was designed for this CPS type as well. Primers were designed in regions that were found to be unique to each particular serotype (Table 3), as described in Materials and Methods. Each primer set was tested on the strain from which it was designed and the 23 strains shown in Table 1 to confirm specificity for these serotypes. Primer sets were judged to be satisfactory if they produced the expected size PCR product on their Penner serotype DNA template or related complexes and were negative for the other tested serotypes. Data are shown in Table S2 in the supplemental material.

Next the primer sets were grouped into two mixes on the basis of the sizes of the products (Table 3). The alpha mix contained primers that distinguish HS2, the HS3 complex, HS6, HS10, HS15, HS41, HS53, and part of the HS4 complex (HS4 and HS13, termed HS4A). The beta mix contains primers that distinguish the HS1 complex (including HS1 and HS1/44), the HS23/36 complex, the HS8 complex (HS8 and HS17), HS42, HS44, and part of the HS4 complex (HS4/13/64 or CG8486-like termed HS4B). Following amplification, the alpha and beta mix PCRs were run separately on a 2% agarose gel in parallel with a 100-bp ladder to decipher the capsule type. Amplicon sizes ranged from 62 bp for HS2 to 652 bp for HS4B (Fig. 3). Expected sizes are listed in Table 3.

Validation of multiplex PCR. To validate the multiplex PCR, the alpha and beta mixes were tested on 244 serotyped strains from Thailand and Egypt. The results are summarized in Table 4. These strains included 184 strains that typed as one of the CPS types included in the multiplex, 37 strains that typed as serotypes that were not included in the multiplex, and 23 strains that were not typeable in the Penner scheme.

Overall, the multiplex method had a specificity and accuracy of >97% and a sensitivity of >90% for the 184 strains that serotyped as 1 of the 17 serotypes covered in the multiplex (Table 4). The method detected 100% of strains of HS2 (30/30), HS8/17 (10/10), HS15 (19/19), HS23/36 (13/13), HS41 (2/2), HS53 (16/16), and HS6 (1/1). There were two false-negative results with each of the HS1/44 primers (2/25), the HS3 complex primers (2/26), and the HS4 complex primers (2/20). The HS10 primers resulted in one false-negative result (1/14) and two false-positive results (one HS1/44 and one HS44 serotype). The HS6 primers picked up five strains, two of which belonged to the HS3 complex, and the HS15 primers picked up 6 false-positive results, all of which were HS31 (see below).

There were 37 strains tested that belonged to 15 serotypes not included in the multiplex. A total of 27 of these 37 strains were negative with the multiplex primers, but 6 HS31 strains reacted with the HS15 primers, as discussed above. This might suggest that HS31 and HS15 share some genes and that primers may need to be redesigned to distinguish these serotypes. There were also individual strains that typed as HS32, HS35, and HS59 that reacted with the HS6 primers, and one HS37 strain reacted with the HS3 primers. Again, HS32, HS35, HS37, and HS59 strains have not been characterized, and the significance of these results will require additional investigation. Of the 23 strains that were untypeable in the Penner scheme, 8 reacted with the HS6 primers, 2 reacted with the HS15 primers, and 1 each reacted with the HS2 and HS10 primers. These data suggest that some strains are untypeable in the Penner scheme because the phase-variable CPS was not being expressed in the colonies selected for serology.

Conclusions. The potential of a CPS conjugate vaccine against this pathogen offers a novel solution to the problem of *C. jejuni*-induced diarrhea for travelers and residents of areas where it is endemic, but determination of major CPS types in areas of endemicity is critical to development of such a vaccine. Classical Penner serotyping is based primarily on CPS, although other structures (e.g., LOS) have been shown to confer serospecificity in some cases (18, 39). Penner typing is time and labor-intensive and is currently performed in only a few labs worldwide. The availability of a multiplex PCR method to determine CPS type overcomes many of the complexities of the Penner scheme, and the method also does not require that the phase-variable CPS be expressed at the time of testing. In this study, we demonstrated the possibility of specifically rec-

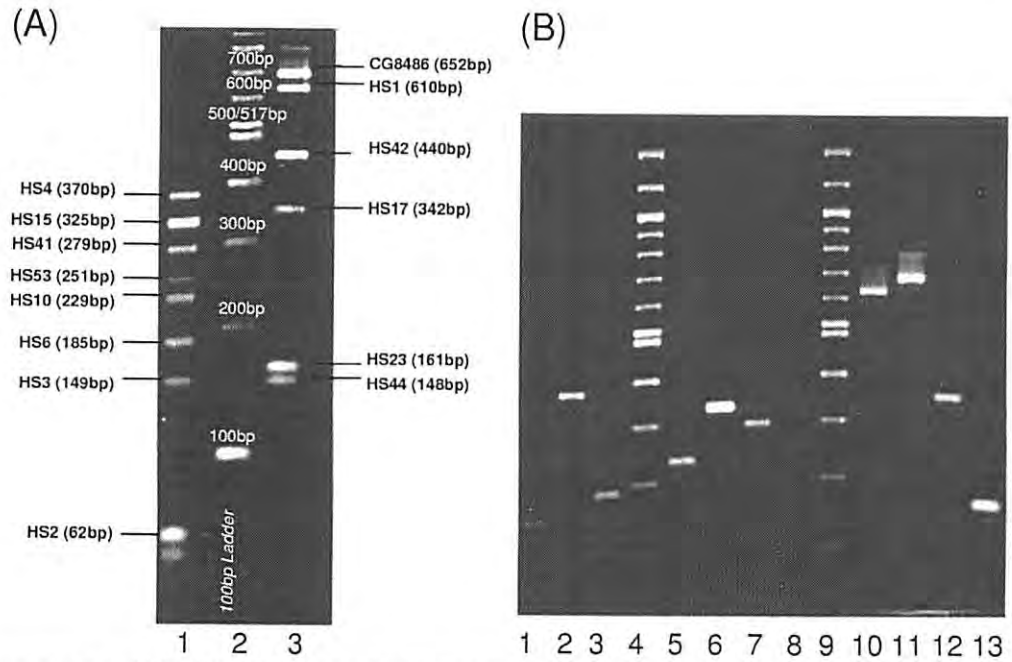

FIG. 3. Representative multiplex PCR with alpha and beta mixes separated on 2% agarose. (A) Lane 1, mixture of PCR products amplified with the alpha primer sets on HS4A, HS15, HS41, HS53, HS10, HS6, HS3, and HS2 DNAs; lane 2, 100-bp NEB DNA standard; lane 3, mixture of PCR products amplified with the beta primer sets on HS4B, HS1, HS42, HS17, HS23, and HS44 DNAs. Amplicons were separated on 2% agarose as described in Materials and Methods. The position of each CPS-specific amplicon is shown. (B) Typical PCR products obtained with primer mixes alpha and beta. Lane 1, alpha primer sets on HS3 DNA; lane 2, alpha primer sets on HS4 DNA; lane 3, alpha primer sets on HS6 DNA; lane 4, 100-bp NEB DNA standard; lane 5, alpha primer sets on HS10 DNA; lane 6, alpha primer sets on HS15 DNA; lane 7, alpha primer sets on HS41 DNA; lane 8, alpha primer sets on HS53 DNA; lane 9, 100-bp NEB DNA standard; lane 10, PCR beta primer sets on HS1 DNA; lane 11, beta primer sets on HS4/13/64 DNA; lane 12, beta primer sets on HS8 DNA; lane 13, beta primer sets on HS23 DNA.

ognizing CPS types by a multiplex PCR. Although the present system can discriminate some of the complexes observed within the original Penner scheme (e.g., HS4 and HS4/13/64), it has not been designed to break all of the complexes into individual serotypes but rather can distinguish related CPS types. Thus, the differences among the members of the HS23/36 complex are based on changes in phase-variable genes and cannot be discriminated by PCR. Similarly, the minor differences in sequence between HS8 and HS17 could not be distinguished.

Including this study, the CPS loci from 18 different Penner serotypes have been sequenced. Collectively, these data, including those for the 8 loci reported here, reveal a mosaic nature of the CPS genes, which is likely due to horizontal transfer among strains of this naturally transformable organism. Thus, we have demonstrated that HS10 and HS15 are mosaics of HS4 and HS41 and that HS8 and HS17 are mosaics of HS41, HS42, and HS1. We view this multiplex system to be dynamic and expect it to evolve as additional CPS loci are sequenced. The current data suggest that strains within a given

TABLE 4. Validation of CPS multiplex PCR with 244 strains of known Penner type

Primer set	No. of strains with the following result:					% [a]		
	Total	True positive	False positive	False negative	True negative	Accuracy	Sensitivity	Specificity
HS1/44 complex	25	23	0	2	219	99.18	92.00	100.00
HS2	30	30	0	0	214	100.00	100.00	100.00
HS3 complex	26	24	1	2	217	98.77	92.31	99.54
HS4 complex	20	18	0	2	224	99.18	90.00	100.00
HS6	1	1	5	0	238	97.95	100.00	97.94
HS8/17 complex	10	10	0	0	234	100.00	100.00	100.00
HS10	14	13	2	1	228	98.77	92.86	99.13
HS15	19	19	6	0	219	97.54	100.00	97.33
HS23/36 complex	13	13	1	0	230	99.59	100.00	99.57
HS41	2	2	0	0	242	100.00	100.00	100.00
HS42	8	8	0	0	236	100.00	100.00	100.00
HS53	16	16	1	0	227	99.59	100.00	99.56

[a] Accuracy was calculated as follows: (true positive + true negative)/(true positive + true negative + false positive + false negative). Sensitivity was calculated as follows: (true positive)/(true positive + false negative). Specificity was calculated as follows: (true negative)/(true negative + false positive).

complex are highly related and that the differences among some serotypes are due to phase variation of a limited number of genes. This is consistent with older data that showed serotype variations within Penner complexes (7, 24, 26, 39).

The multiplex system, in conjunction with structural analyses of additional CPS polysaccharides, will help refine our understanding of the relationship of strains within the Penner scheme and help define strains that may be immunologically cross-reactive. In another mucosal pathogen for which CPS is a protective antigen, *Streptococcus pneumoniae*, some CPS structures are associated with more severe disease (8, 15). The ability to rapidly determine CPS type may also help determine if specific *C. jejuni* CPS structures are also associated with more severe disease.

ACKNOWLEDGMENTS

We thank Eva Nielsen and Helen Tabor for performing Penner serotyping of strains, Stephen J. Savarino for providing the Egyptian strains, Piyarat Pootong and Panida Nopthai for technical assistance, and Mario A. Monteiro for helpful comments on the manuscript.

This work was supported by U.S. Naval Medical Research and Development Command Work Unit 6000.RAD1.DA3.A0308.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the U.S. government.

P.G., O.S., and C.M. are employees of the U.S. government, and this work was prepared as part of their official duties.

REFERENCES

1. **Allos, B. M.** 2001. Campylobacter jejuni infections: update on emerging issues and trends. Clin. Infect. Dis. **32**:1201–1206.
2. **Ang, C. W., B. C. Jacobs, and J. D. Laman.** 2004. The Guillain-Barre syndrome: a true case of molecular mimicry. Trends Immunol. **25**:61–66.
3. **Aspinall, G. O., C. M. Lynch, H. Pang, R. T. Shaver, and A. P. Moran.** 1995. Chemical structures of the core region of Campylobacter jejuni O:3 lipopolysaccharide and an associated polysaccharide. Eur. J. Biochem. **231**:570–578.
4. **Aspinall, G. O., A. G. McDonald, and H. Pang.** 1992. Structures of the O chains from lipopolysaccharides of Campylobacter jejuni serotypes O:23 and O:36. Carbohydr. Res. **231**:13–30.
5. **Bachtiar, B. M., P. J. Coloe, and B. N. Fry.** 2007. Knockout mutagenesis of the kpsE gene of Campylobacter jejuni 81116 and its involvement in bacterium-host interactions. FEMS Immunol. Med. Microbiol. **49**:149–154.
6. **Bacon, D. J., et al.** 2001. A phase-variable capsule is involved in virulence of Campylobacter jejuni 81-176. Mol. Microbiol. **40**:769–777.
7. **Bradbury, W. C., A. D. Pearson, M. A. Marko, R. V. Congi, and J. L. Penner.** 1984. Investigation of a Campylobacter jejuni outbreak by serotyping and chromosomal restriction endonuclease analysis. J. Clin. Microbiol. **19**:342–346.
8. **Brueggemann, A. B., et al.** 2003. Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype- and clone-specific differences in invasive disease potential. J. Infect. Dis. **187**:1424–1432.
9. **Chen, Y. H., F. Poly, Z. Pukulski, P. Guerry, and M. A. Monteiro.** 2008. The chemical structure and genetic locus of Campylobacter jejuni CG8486 (serotype HS:4) capsular polysaccharide: the identification of 6-deoxy-D-ido-heptopyranose. Carbohydr. Res. **343**:1034–1040.
10. **Fouts, D. E., et al.** 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple Campylobacter species. PLoS Biol. **3**:e15.
11. **Gilbert, M., R. E. Mandrell, C. T. Parker, J. Li, and E. Vinogradov.** 2007. Structural analysis of the capsular polysaccharide from Campylobacter jejuni RM1221. Chembiochem **8**:625–631.
12. **Grant, A. J., et al.** 2005. Signature-tagged transposon mutagenesis studies demonstrate the dynamic nature of cecal colonization of 2-week-old chickens by Campylobacter jejuni. Appl. Environ. Microbiol. **71**:8031–8041.
13. **Griffiths, P. L., and R. W. Park.** 1990. Campylobacters associated with human diarrhoeal disease. J. Appl. Bacteriol. **69**:281–301.
14. **Guerry, P., et al.** 2002. Phase variation of Campylobacter jejuni 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. Infect. Immun. **70**:787–793.
15. **Hanage, W. P., et al.** 2005. Invasiveness of serotypes and clones of Streptococcus pneumoniae among children in Finland. Infect. Immun. **73**:431–435.
16. **Hanniffy, O. M., et al.** 1999. Chemical structure of a polysaccharide from Campylobacter jejuni 176.83 (serotype O:41) containing only furanose sugars. Carbohydr. Res. **319**:124–132.
17. **Hendrixson, D. R.** 2006. A phase-variable mechanism controlling the Campylobacter jejuni FlgR response regulator influences commensalism. Mol. Microbiol. **61**:1646–1659.
18. **Karlyshev, A. V., et al.** 2005. Analysis of Campylobacter jejuni capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. Mol. Microbiol. **55**:90–103.
19. **Karlyshev, A. V., D. Linton, N. A. Gregson, A. J. Lastovica, and B. W. Wren.** 2000. Genetic and biochemical evidence of a Campylobacter jejuni capsular polysaccharide that accounts for Penner serotype specificity. Mol. Microbiol. **35**:529–541.
20. **Korlath, J. A., M. T. Osterholm, L. A. Judy, J. C. Forfang, and R. A. Robinson.** 1985. A point-source outbreak of campylobacteriosis associated with consumption of raw milk. J. Infect. Dis. **152**:592–596.
21. **McNally, D. J., et al.** 2006. The HS:19 serostrain of Campylobacter jejuni has a hyaluronic acid-type capsular polysaccharide with a nonstoichiometric sorbose branch and O-methyl phosphoramidate group. FEBS J. **273**:3975–3989.
22. **McNally, D. J., et al.** 2005. The HS:1 serostrain of Campylobacter jejuni has a complex teichoic acid-like capsular polysaccharide with nonstoichiometric fructofuranose branches and O-methyl phosphoramidate groups. FEBS J. **272**:4407–4422.
23. **McNally, D. J., et al.** 2007. Commonality and biosynthesis of the O-methyl phosphoramidate capsule modification in Campylobacter jejuni. J. Biol. Chem. **282**:28566–28576.
24. **Melby, K., G. Storvold, R. V. Congi, and J. L. Penner.** 1985. Serotyping of Campylobacter jejuni isolated from sporadic cases and outbreaks in northern Norway. Acta Pathol. Microbiol. Immunol. Scand. B **93**:83–86.
25. **Miller, W. G., et al.** 2000. Detection on surfaces and in Caco-2 cells of Campylobacter jejuni cells transformed with new gfp, yfp, and cfp marker plasmids. Appl. Environ. Microbiol. **66**:5426–5436.
26. **Mills, S. D., B. Kuzniar, B. Shames, L. A. Kurjanczyk, and J. L. Penner.** 1992. Variation of the O antigen of Campylobacter jejuni in vivo. J. Med. Microbiol. **36**:215–219.
27. **Monteiro, M. A., et al.** 2009. Capsule polysaccharide conjugate vaccine against diarrheal disease caused by Campylobacter jejuni. Infect. Immun. **77**:1128–1136.
28. **Muldoon, J., et al.** 2002. Structures of two polysaccharides of Campylobacter jejuni 81116. Carbohydr. Res. **337**:2223–2229.
29. **Oberhelman, R. A., and D. N. Taylor.** 2000. Campylobacter infections in developing countries, p. 139–154. *In* Campylobacter, 2nd ed. American Society for Microbiology, Washington, DC.
30. **Palmer, S. R., et al.** 1983. Water-borne outbreak of campylobacter gastroenteritis. Lancet **i**:287–290.
31. **Parkhill, J., et al.** 2000. The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. Nature **403**:665–668.
32. **Pearson, B. M., et al.** 2007. The complete genome sequence of Campylobacter jejuni strain 81116 (NCTC11828). J. Bacteriol. **189**:8402–8403.
33. **Penner, J. L., and J. N. Hennessy.** 1980. Passive hemagglutination technique for serotyping Campylobacter fetus subsp. jejuni on the basis of soluble heat-stable antigens. J. Clin. Microbiol. **12**:732–737.
34. **Penner, J. L., J. N. Hennessy, and R. V. Congi.** 1983. Serotyping of Campylobacter jejuni and Campylobacter coli on the basis of thermostable antigens. Eur. J. Clin. Microbiol. **2**:378–383.
35. **Pimentel, M., et al.** 2008. A new rat model links two contemporary theories in irritable bowel syndrome. Dig. Dis. Sci. **53**:982–989.
36. **Poly, F., et al.** 2007. Genome sequence of a clinical isolate of Campylobacter jejuni from Thailand. Infect. Immun. **75**:3425–3433.
37. **Poly, F., et al.** 2008. Characterization of two Campylobacter jejuni strains for use in volunteer experimental-infection studies. Infect. Immun. **76**:5655–5667.
38. **Pope, J. E., A. Krizova, A. X. Garg, H. Thiessen-Philbrook, and J. M. Ouimet.** 2007. Campylobacter reactive arthritis: a systematic review. Semin. Arthritis Rheum. **37**:48–55.
39. **Preston, M. A., and J. L. Penner.** 1989. Characterization of cross-reacting serotypes of Campylobacter jejuni. Can. J. Microbiol. **35**:265–273.
40. **Rachlin, J., C. Ding, C. Cantor, and S. Kasif.** 2005. MuPlex: multi-objective multiplex PCR assay design. Nucleic Acids Res. **33**:W544–W547.
41. **Rao, M. R., et al.** 2001. Pathogenicity and convalescent excretion of Campylobacter in rural Egyptian children. Am. J. Epidemiol. **154**:166–173.
42. **Rutherford, K., et al.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16**:944–945.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
44. **Spiller, R. C.** 2007. Role of infection in irritable bowel syndrome. J. Gastroenterol. **42**(Suppl. 17):41–47.
45. **Szymanski, C. M., et al.** 2003. Detection of conserved N-linked glycans and phase-variable lipooligosaccharides and capsules from campylobacter cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. J. Biol. Chem. **278**:24509–24520.
46. **Vallone, P. M., and J. M. Butler.** 2004. AutoDimer: a screening tool for primer-dimer and hairpin structures. Biotechniques **37**:226–231.
47. **Waldenstrom, J., et al.** 2010. Campylobacter jejuni colonization in wild birds: results from an infection experiment. PLoS One **5**:e9082.